Geographical differentiation in floral traits across the distribution range of the Patagonian oil-secreting *Calceolaria polyrhiza*: do pollinators matter?

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• *Background and Aims* The underlying evolutionary processes of pollinator-driven floral diversification are still poorly understood. According to the Grant–Stebbins model speciation begins with adaptive local differentiation in the response to spatial heterogeneity in pollinators. Although this crucial process links the micro- and macroevolution of floral adaptation, it has received little attention. In this study geographical phenotypic variation was investigated in Patagonian *Calceolaria polyrhiza* and its pollinators, two oil-collecting bee species that differ in body size and geographical distribution.
• *Methods* Patterns of phenotypic variation were examined together with their relationships with pollinators and abiotic factors. Six floral and seven vegetative traits were measured in 45 populations distributed across the entire species range. Climatic and edaphic parameters were determined for 25 selected sites, 2–16 bees per site of the most frequent pollinator species were captured, and a critical flower–bee mechanical fitting trait involved in effective pollination was measured. Geographical patterns of phenotypic and environmental variation were examined using uni- and multivariate analyses. Decoupled geographical variation between corolla area and floral traits related to the mechanical fit of pollinators was explored using a Mantel test.
• *Key Results* The body length of pollinators and the floral traits related to mechanical fit were strongly correlated with each other. Geographical variation of the mechanical-fit-related traits was decoupled from variation in corolla size; the latter had a geographical pattern consistent with that of the vegetative traits and was mainly affected by climatic gradients.
• *Conclusions* The results are consistent with pollinators playing a key role in shaping floral phenotype at a geographical scale and promoting the differentiation of two floral ecotypes. The relationship between the critical floral-fit-related trait and bee length remained significant even in models that included various environmental variables and an allometric predictor (corolla area). The abiotic environment also has an important role, mainly affecting floral size. Decoupled geographical variation between floral mechanical-fit-related traits and floral size would represent a strategy to maintain plant–pollinator phenotypic matching in this environmentally heterogeneous area.

Key words: Abiotic environmental gradients, bee morphology, *Calceolaria*, floral ecotypes, geographical range, local adaptation, oil-collecting bees, oil-offering flowers, Patagonia, phenotypic covariance, specialized pollination, speciation, vegetative morphology.

INTRODUCTION

Traditionally the great diversification of animal-pollinated angiosperms has been proposed to be promoted by divergence in pollinators (Darwin, 1862; Grant and Grant, 1965; Johnson, 2010). However, the underlying evolutionary and ecological processes are still poorly understood, in part because most studies have focused on floral diversification and pollination relationships in a phylogenetic context or pollinator-mediated phenotypic selection within populations (reviewed in Herrera *et al.*, 2006; Johnson, 2006). Exploring adaptive intraspecific floral differentiation in response to pollinator geographical variation is crucial in understanding this macroevolutionary pattern (Gould and Johnston, 1972; Herrera *et al.*, 2006). A conceptual model of pollinator-driven speciation first developed by Grant and Grant (1965) and Stebbins (1970) postulated that speciation begins with adaptation by plants to their most effective pollinators at a local scale (see also Johnson, 2006). Thus, given a geographical mosaic of pollinator availability, one would expect that floral morphology would diverge as plants become locally adapted to respective pollinator variants (i.e. 'pollination ecotypes'; Herrera *et al.*, 2006; Johnson, 2006).

Recent studies of geographical variation of phenotypic traits have provided insights into the role of pollinators as major drivers of evolutionary differentiation (e.g. Johnson, 2006; Anderson and Johnson, 2008, 2009; Pauw *et al.*, 2009; Peter and Johnson, 2014; Van der Niet *et al.*, 2014). However, little is known about the relative contributions of other factors in the geographical differentiation of floral traits involved in plant–pollinator interactions (Herrera *et al.*, 2006; Kay and Sargent, 2009). Several studies of abiotic factors account for the influence of climate and soil on floral phenotype (e.g. Galen, 1999; Carroll *et al.*, 2001; Herrera, 2005). Moreover, some floral traits involved in plant–pollinator interaction, particularly those related to attraction (i.e. corolla size) and floral reward, in general demand large amounts of water and nutrients (Galen, 1999), and thus

may vary geographically due to resource reallocation under stressful conditions (e.g. Jonas and Geber, 1999; Carroll *et al.*, 2001, Herrera, 2005; Strauss and Whittall, 2006). For example, in areas with limited water and soil nutrient availability it is generally advantageous to produce smaller flowers as a strategy to reduce evapotranspiration and decrease the amount of resources allocated to corolla tissue (Galen, 1999; Carroll *et al.*, 2001; Herrera, 2005, 2009).

However, few studies that considered floral trait evolution in a geographical context have taken into account the influence of pollinators concomitantly with abiotic factors (but see Anderson and Johnson, 2008; Pérez-Barrales *et al.*, 2007, 2009). Moreover, because vegetative traits play a key role in the energy and water balance of the plant (e.g. Rico-Gray and Palacios-Rios, 1996; Del Pozo *et al.*, 2002; Roche *et al.*, 2004) and because they are not directly subjected to pollinator-mediated selection (Armbruster *et al.*, 1999; Totland, 2001; Herrera *et al.*, 2002), exploring covariation patterns between floral and vegetative traits might demonstrate that the observed floral variation pattern could not be attributed directly to selection pressure mediated by pollinators (Herrera, 2005; Lambrecht and Dawson, 2007; Pérez-Barrales *et al.*, 2009). Despite these considerations, to our knowledge very few studies have included abiotic factors and vegetative characters in the context of floral evolution at a broad spatial scale (but see Anderson and Johnson, 2008; Chalcoff *et al.*, 2008). Widely distributed species with specialized pollination systems provide the opportunity to investigate the possible role of pollinators and abiotic factors in shaping floral phenotypes.

In plants with specialized pollination, functionally important traits related to plant–pollinator morphological fit are expected to be under strong selection for accuracy (e.g. Armbruster *et al.*, 2009). However, this may be in conflict with the general tendency for size-related traits to covary (e.g. corolla size) along climatic and edaphic gradients (e.g. Lambrecht and Dawson, 2007). Decoupling of phenotypic variance between mechanical-fit-related traits and other floral traits, such as corolla size, could be a strategy to facilitate plant–pollinator phenotypic matching across a variable landscape.

The degree of covariation of population means of different morphological characters can be used to assess the degree to which traits are correlated as a result of evolutionary divergence of populations (Armbruster 1990, 1991). Consequently, strong correlations at inter-population level are likely to reflect the shape of the underlying fitness surface governing floral differentiation across the geographical range of a species (Armbruster *et al.*, 2004). However, traits that are developmentally related would also be correlated and would have evolved as a unit (e.g. Cheverud *et al.*, 1989; Armbruster *et al.*, 2004). Therefore, testing functional and developmental hypotheses is an essential means of providing evidence for the processes acting on character covariation at a geographical scale (Cheverud *et al.*, 1989).

Oil-secreting flowers and oil-collecting bees represent one of the most specialized plant–pollinator systems among the angiosperms (e.g. Vogel, 1988; Johnson and Steiner, 2000; Sérsic, 2004; Pauw, 2006; Cosacov *et al.*, 2008). Flowers offering fatty oils are only found in ten families of flowering plants (Buchmann, 1987; Neff and Simpson, 2005). These flowers are visited by highly specialized bees, which constitute only 1·4 % of the bee species in the world (Buchmann, 1987; Cocucci *et al.*, 2000).

Calceolaria polyrhiza is a xenogamous perennial herb endemic to Patagonia (Molau, 1988; Ehrhart, 2000). Like many of its congeners, it produces non-volatile oils as floral rewards that attract specialized oil-collecting solitary bees. Across its distribution range, *C. polyrhiza* is exclusively pollinated by two oil-collecting bee species, *Chalepogenus caeruleus* (Tribe Tapinotaspidini) and *Centris cineraria* (Tribe Centridini; Simpson and Neff, 1981; Roig-Alsina, 1999, 2000; Sérsic, 2004; Cosacov, 2010), which greatly differ in their distribution ranges, body length and oil-collecting behaviour (Molau, 1988; Roig-Alsina, 1999, 2000; Sérsic, 2004; Cosacov, 2010). Previous studies of the reproductive biology of *Calceolaria* suggest an important morphological correspondence between flowers and pollinators (Sérsic, 2004). Moreover, the relationship between pollinators and floral variation in a phylogenetic context in *Calceolaria* suggests that plant–pollinator interaction would be an important factor in genus diversification (Cosacov *et al.*, 2009).

Here we explore the relationship between pollinator shifts and floral trait variation in *C. polyrhiza* across a broad geographical scale, while simultaneously accounting for variation in climatic and edaphic factors. We ask the following specific questions: (1) What is the extent of environmental variation (i.e. biotic and abiotic factors) among populations? (2) What is the extent of floral and vegetative variation among populations? (3) What is the degree of coupling/decoupling between vegetative and floral traits, and among floral traits? (4) What are the most probable drivers of variation (biotic versus abiotic factors) in floral and vegetative attributes? By answering these questions we seek to approach a more fundamental issue: the relative importance of pollinators and abiotic factors as drivers of floral diversity patterns.

MATERIALS AND METHODS

Study species and sites

Calceolaria polyrhiza (Calceolariaceae) is distributed in Argentina from southern Mendoza Province (35°S) to southern Santa Cruz Province (52°S). It is found from sea level to 3000 m a.s.l. and under diverse climatic conditions. In Chile, *C. polyrhiza* is less abundant and is found in scattered locations from 35° S to 45°S. It has hermaphroditic flowers with two stamens and a two-lipped corolla with a distinctive inflated lower lip bearing a median lobe, the appendage or lap (Sérsic, 2004). This lip is folded inwards and carries the elaiophore, a patch of oil-secreting trichomes. Like most species in this genus, *C. polyrhiza* provides oils as rewards to pollinators (Vogel, 1974; Molau, 1988; Sérsic, 2004). It has a predominantly outcrossing mating system and flowers from mid-October to early December (Cosacov, 2010). Because seeds are dispersed mainly by gravity (Molau, 1988; Fernández *et al.*, 2002), the mode of dispersal is very limited and pollinator movements are spatially confined (<100 m; Sérsic, 2004; Michener, 2007), gene flow among populations via seeds and pollen is presumably limited.

A total of 45 populations of *C. polyrhiza* were surveyed over five consecutive flowering seasons (2004–2008). Thirty-eight populations were located in Argentina and seven in Chile,

covering the entire species distribution range (a latitudinal extension of 2375 km) and the two main ecological regions where the species grows, the arid Patagonian steppe and the understorey of Andean–Patagonian forests (Fig. 1, Supplementary Data Table S1). The Patagonian steppe is a large (673 000 km²), dry, extra-Andean plain covered by grassland and scrubby vegetation that extends from the eastern slopes of the southern Andes to the Atlantic coast. The Andean–Patagonian forest is a much smaller area (248 100 km²) covered by woodlands that extends from 35°S to 55°S on the eastern and western slopes of the Andes, reaching the western edge of the Patagonian steppe to the east.

Floral and vegetative traits measured

In each of the 45 populations sampled, 10–51 individuals were randomly chosen, separated by at least 5 m to avoid collecting clones or close relatives. One to three freshly opened flowers (i.e. flowers with bright colours, anthers with pollen, and undamaged corolla) and three fully expanded leaves per individual were collected at random (from a total of 1231 individuals). Six floral traits were measured (Fig. 2, Appendix 1): style length (SL), filament length (FL), theca length (TheL), throat length (ThroL), elaiophore width (EW) and corolla area (CA). Throat length is the 'operative distance', i.e. the distance between the floral
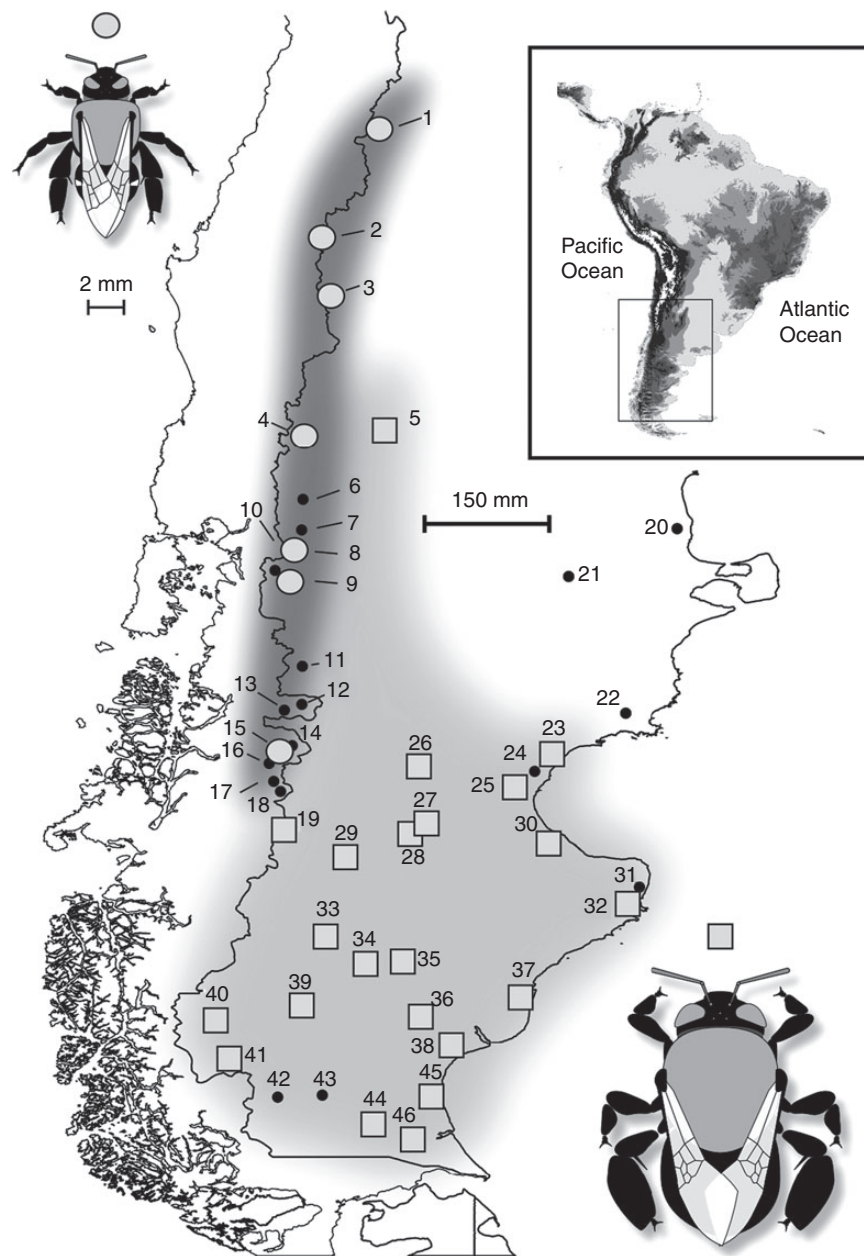

FIG. 1. Map of the study area showing the 45 *Calceolaria polyrhiza* populations sampled. Populations pollinated by *Chalepogenus caeruleus* (grey circles) or *Centris cineraria* (grey squares) or those where pollinators were not observed (black dots) are also indicated (based on Cosacov, 2010). Dark and light shading indicate *C. polyrhiza* populations located in Andean-Patagonian forest or in the extra-Andean plain, respectively. Locality numbers correspond to those in Appendix 1 (N$_{loc}$). The inset depicts a shaded relief map of South America with the study area indicated with a box.
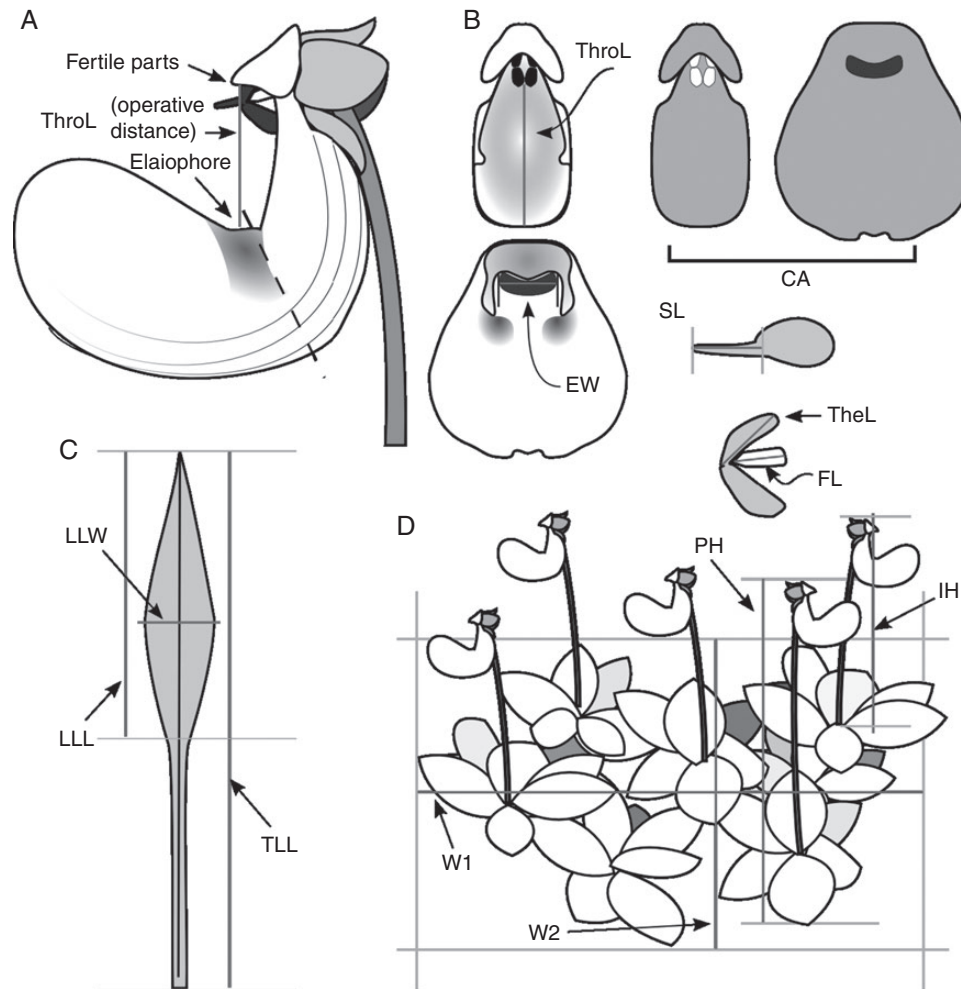
FIG. 2. Schematic diagram of a flower, leaf and plant of *Calceolaria polyrhiza* showing linear morphometric measurements. (A) Lateral view of a flower showing fertile parts, elaiophore and the distance between the floral reward (elaiophore) and the fertile parts, i.e. throat length (ThroL). Black dashed line indicates the point where flowers were dissected to obtain parts shown in (B). (B) Floral traits: throat length (ThroL), elaiophore width (EW), corolla area (CA) indicated by the black surface, theca length (TheL), filament length (FL) and style length (SL). (C) Vegetative traits: total leaf length (TLL), leaf lamina length (LLL), leaf lamina width (LLW). (D) Plant traits: inflorescence height (IH), plant height (PH), plant maximum width (W1) and perpendicular width (W2).

reward (i.e. the elaiophore) and the fertile parts; therefore, this is the distance that pollinators have to fit to efficiently transfer pollen from the anthers and to deposit it on the stigmas (Fig. 3). Three foliar traits were measured (Fig. 2, Appendix 2): total leaf length including petiole (TLL), leaf lamina width (LLW) and leaf lamina length (LLL). We calculated TLL × LLW as an estimator of total leaf area (TLA), LLL × LLW as an estimator of leaf lamina area (LLA) and LLL/LLW (L/W) as an estimator of leaf shape. Specific leaf area (SLA) was also calculated as leaf area per unit of dry mass (Cornelissen *et al.*, 2003). To determine leaf dry mass, leaves were dried at 60 °C for 72 h and then weighed to the nearest 0·1 mg using a precision balance (Sartorius CP224 S). Three plant attributes were measured *in situ* (Fig. 2, Appendix 2): plant height (PH), inflorescence height (IH) and plant area (PA), calculated as the product of maximum plant width and perpendicular width. All morphometric measurements of flowers ($n = 3134$) and leaves ($n = 3591$) were taken from digital images using SigmaScan Pro 5·0 software (SPSS). To obtain these images, scaled flowers were placed in a cylindrical case and photographed
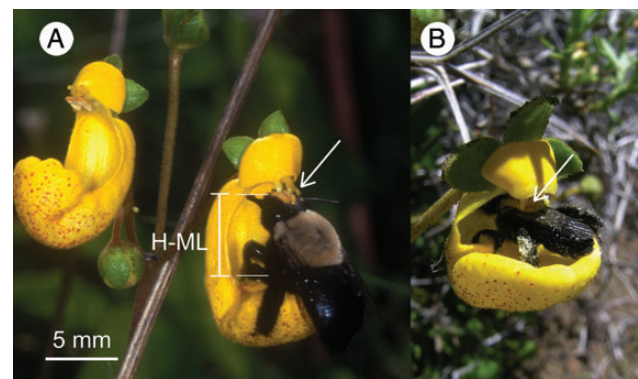
FIG. 3. Oil-collecting bee species pollinating the flowers of *Calceolaria polyrhiza*. (A) The large bee *Centris cineraria* seizing the flower with its mandibles and mid-legs, and collecting oil with the foreleg. Note the stigma and stamens making contact with the dorsal part of the bee's head (arrow). The critical trait involved in effective pollination, distance between the head and the middle legs (H-ML), is indicated. (B) The small bee *Chalepogenus caeruleus*. Note the stigma and stamens making contact with the dorsal part of the bee's thorax (arrow) and the pellet of pollen and oils accumulated on the hind legs.

with a Nikon Coolpix 5400 camera, in frontal and lateral views, and dissected as indicated in Fig. 2. Scale leaves were scanned with 300 d.p.i. resolution.

Climatic and edaphic factors

We recorded geographical coordinates (i.e. latitude and longitude) for each population using a GPS system. From this data set, we selected 25 populations representative of the geographical distribution of the species. Using information available from WorldClim database (http://www.worldclim.org/; Hijmans *et al.*, 2005), bioclimatic variables were obtained for each locality at a spatial resolution of $1 \, \text{km}^2$. From the available information we selected annual temperature and precipitation averages, temperature of the warmest and coldest quarters (i.e. 3-month seasons), and precipitation of the driest and wettest quarters (Supplementary Data Table S2). These variables correspond to average values obtained from interpolations of data observed during the period between the years 1950 and 2000. To characterize edaphic conditions, one soil sample of 500 g was taken from the ground surface to a depth of 15 cm at each location. For each sample, the pH value, organic carbon (C), nitrogen (N), potassium (K), phosphorus (P) and total sand/silt content were determined by the soil laboratory of the Consejo Agrario Provincial, Convenio INTA-CAP, Santa Cruz Province, Argentina.

Pollinators

Calceolaria polyrhiza interacts with two pollinator species across its geographical range, *Chalepogenus caeruleus*, distributed from southern Mendoza Province (35 ° S) to northern Chubut Province (43 °S) and inhabiting the northern area of the Andean–Patagonian forest, and *Centris cineraria*, distributed from southern Neuquén Province (40 ° S) to southern Santa Cruz Province (51 °S) and inhabiting mainly the Patagonian steppe ecoregion (Figs 1 and 3; Roig-Alsina, 1999, 2000; Cosacov, 2010). Both are oil-collecting bee species, endemic to Patagonia (Roig-Alsina, 1999, 2000). In the forest ecoregion, *C. cineraria* was observed in two localities, but records of pollinator visits indicated that *Ch. caeruleus* accounted for >92 % of the total visits recorded in each of these two populations (Cosacov, 2010). A previous study indicates that the localities where *Ch. caeruleus* and *C. cineraria* are distributed differed in climatic conditions (mean annual temperature and precipitation, and mean temperature and precipitation of the warmest quarter) and in edaphic factors (pH value and soil silt content; Cosacov, 2010). To characterize geographical variation in the pollinator functional trait in the 25 *C. polyrhiza* populations selected as described in the section Climatic and edaphic factors, pollinator bees were captured and the distance between the head and the middle legs (HML) was measured under a stereomicroscope using a digital calliper (Appendix 3). This is a critical trait involved in effective pollination, because it fits ThroL (Fig. 3). We explored whether HML had a geographical pattern by using multiple regression models with geographical coordinates. To partition the total variance of HML according to its differences between bee species, among populations within species, and among individuals within populations (the last level was used as the error term; Sokal and Rohlf, 1995), a mixed-effects nested ANOVA was performed for this trait; the

percentage of variance accounted for each level, the component of variance (CV), was then estimated.

Statistical analyses

Structure of geographical variation in floral and vegetative traits. To partition the total variance of floral and vegetative traits into its hierarchical components, a mixed-effects nested ANOVA was performed for each trait measured. Floral and vegetative attributes were partitioned according to their differences among populations, among individuals within populations, and among flowers or leaves within individuals (the latter level was used as the error term; Sokal and Rohlf, 1995). Although not all populations were sampled in the same year, during each flowering season we sampled populations representing the entire distribution range of the species. Thus, a possible year effect was uniformly distributed across the geographical range. In addition, two populations surveyed over 2 years did not show a temporal effect in morphological variation ($P > 0.05$; Cosacov, 2010). To assess the presence of distance-based patterns of variation in floral and vegetative phenotypes, a spatial autocorrelation analysis was performed for each floral and vegetative trait. Autocorrelograms were constructed using distance classes of 100 km. Significance levels of Moran's *I* coefficients of spatial autocorrelation were obtained by Monte Carlo methods with 999 simulations. These analyses were performed using the library ncf of the R package (R Development Core Team, 2010). Finally, we explored geographical patterns in individual floral and vegetative traits using simple regression models with geographical coordinates. Because precipitation and the edaphic gradient follow a non-linear pattern with longitude in the study area (Paruelo *et al.*, 1998; Cosacov, 2010), we explored whether a non-linear adjustment would give a better fit than a linear one.

Coupled/decoupled geographical variation among floral traits. We used the approach proposed by Cheverud *et al.* (1989) to evaluate whether CA was more strongly correlated with vegetative traits than with floral ones, and whether floral mechanical-fit-related traits (ThroL, SL, FL) were more closely associated among themselves than with the remaining floral traits, across the geographical range of the studied species. The approach assumes that traits that are functionally and/or developmentally related would be correlated and would have evolved as a unit (e.g. Cheverud *et al.*, 1989). Basically, the approach uses two types of matrix: a hypothetical matrix that describes theoretical relationships among traits, and a matrix containing the empirically derived morphometric correlations (Pearson product–moment correlations between all pairs of morphological variables using population mean values of each trait measured). In the simplest case of a matrix of theoretical relationships, characters were identified as linked (indicated as 0·5) or unlinked (indicated as 0). Thus, we constructed two hypothetical matrices of developmental (d) and functional (f) relationships for the whole plant (including floral and vegetative traits) and only for flowers (to further explore coupling/decoupling among floral traits). For the whole plant, we considered two developmental domains (vegetative and floral); when we considered only flowers, we distinguished three developmental domains (corolla, androecium and gynoecium; Supplementary Data Fig. S1). Additionally,

for the whole plant and for flowers alone, we constructed a more complex model: nested hypotheses (n) with hierarchical linkage. In the nested hypotheses two characters were identified as developmentally linked (indicated as 0·5) or unlinked (indicated as 0). Then, if the characters were assumed to be functionally related, an additional linkage value of 0·5 was added to the fundamental developmental linkage. Thus, if two traits were not linked either by development or by function the theoretical relationship assumed was zero; if two traits were linked only by development or function, the relationship assumed was 0·5; and if two traits were linked only by development and function, the theoretical relationship was 1 (Supplementary Data Fig. S1). We then tested which of two alternative theoretical matrices best fitted the empirical matrix and whether their difference in fit was statistically significant. For this purpose, a Z matrix was obtained from the difference of the two competing theoretical matrices, after standardizing the elements of both matrices to zero mean and unity standard deviation. The pattern of similarity between the theoretical and empirical matrices was subjected to Mantel tests with 999 permutations, implemented in the package Vegan of the R 2·15·2 statistical software (R Development Core Team, 2010).

Multivariate patterns and possible factors of morphological variation. To analyse the relationship between floral traits and their association with biotic (i.e. pollinator size) and abiotic (climatic and edaphic) factors, a multivariate analysis of redundancy (RDA) was performed using CANOCO (ter Braak and Šmilauer, 2002). RDA is a multivariate ordination method in which the axes are constructed by a linear combination of environmental variables (ter Braak and Šmilauer, 2002). For this analysis, a matrix of population × morphology was analysed in relation to a corresponding matrix of explanatory environmental variables. We included five explanatory factors that were hypothesized to be important determinants of variation in flower morphology: two edaphic, precipitation and temperature axes derived from their respective principal component analyses (see Climatic and edaphic factors in the Results section) and the critical distance measured on captured pollinators (HML). When necessary, variables were log-transformed. To avoid using different scales and for comparative purposes, the variables were standardized to zero mean and unity standard deviation. The significance of the variability explained by each environmental factor was analysed by automatic selection of variables using a Monte Carlo test with 999 permutations. In this procedure, the variable that best fits the data is selected first and then the next best fitting variable is added to the model (ter Braak and Šmilauer, 2002). We also performed an RDA for the vegetative trait matrix using the same five explanatory factors (including bee functional trait and abiotic variables).

Plant–pollinator matching. To further explore plant–pollinator phenotypic matching, we used multiple regression to analyse simultaneously the effects of biotic (pollinator functional trait) and abiotic (climatic and edaphic parameters) predictor variables on ThroL and the relative importances of these effects. Because ThroL might be explained by allometric relationships among floral traits, we also included CA as a predictor variable. As a control test we also performed multiple regression to analyse simultaneously the effects of biotic (pollinator functional trait), abiotic (climatic and edaphic parameters) and ThroL as predictor variables on CA and the relative importances of these effects.

RESULTS

Geographical structure of floral and vegetative trait variation

Mixed-effects models performed on each phenotypic variable indicated that all variables measured differed significantly among populations and, except for the floral trait ThroL and the vegetative traits SLA and leaf shape (L/W), floral and vegetative traits also differed significantly among individuals within populations (Fig. 4A). The degree of phenotypic variability explained by the different hierarchical levels analysed (populations and individuals) varied among the traits studied (Fig. 4A). Except for plant area (PA), among-population differences in the remaining traits explained a larger proportion of variance than differences among individuals within populations. The ratio between variance components among and within populations suggests a predominance of geographical over local factors structuring floral and vegetative variation (Fig. 4B). The critical floral trait ThroL showed the highest variation at a geographical scale.

Spatial autocorrelation analyses show that similarities in almost all vegetative (IH, PH, TLA, LLA, SLA) and floral (TheL, ThroL, SL, FL, CA) traits tended to decrease with increasing distance among populations (Fig. 5A). Populations located at distances <500 km from each other generally had similar floral and vegetative phenotypes (i.e. positive Moran's *I* coefficients), but at longer distances (>500 km) populations had significantly dissimilar phenotypes (i.e. significant and negative Moran's *I* coefficients; Fig. 5A). The correlogram of the vegetative trait SLA and the floral traits CA, SL, FL and ThroL were monotonic decreasing, and nearly all autocorrelation values were significant (Fig. 5A). Similarities in EW, PA and L/W did not show a significant distance-based pattern (results not shown).

The geographical patterns shared by individual morphological traits are shown in Fig. 5B (see also Supplementary Data Fig. S2). Regarding latitudinal patterns, the vegetative traits PA and SLA and the floral traits TheL and CA decreased southwards. Conversely, the mechanical-fit-related traits ThroL, FL and SL increased southwards. On the other hand, variation patterns in vegetative traits IH, PH, LLA and SLA and the floral trait CA were fitted to a quadratic model with longitude (Fig. 5B). EW did not correlate with latitude or longitude, whereas L/W fitted a quadratic model with latitude ($R^2 = 0.27$, $P = 0.002$).

Coupled/decoupled geographical variation among floral traits

Correlation patterns among the floral traits measured are shown in Fig. 6A. The three hypothetical relationships for the whole-plant characters (vegetative and floral traits) assuming developmental, functional or nested developmental–functional linkage fitted the empirical correlation matrix significantly, the strongest correlation being with the functional hypothesis among the three hypotheses tested (Fig. 6B). However, when comparing these alternative hypotheses, they did not differ significantly in their power to explain the empirical matrix. For the flower data set, the functional hypothesis was the only one that significantly explained the empirical matrix (Fig. 6B).
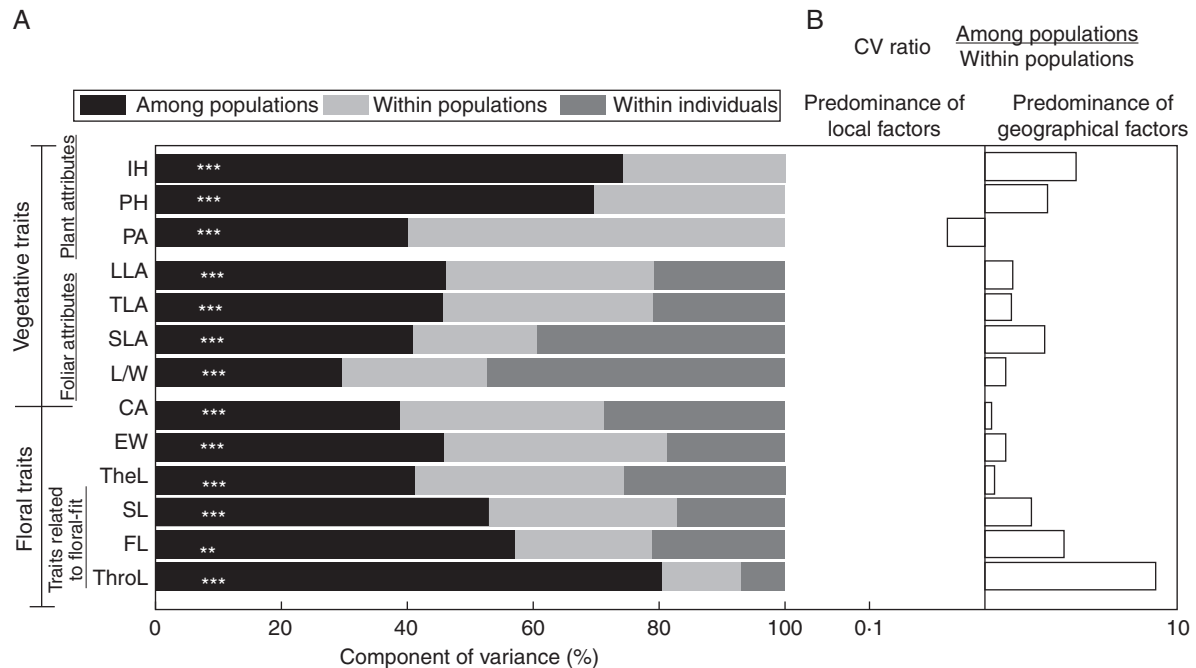
FIG. 4. Structure of phenotypic variation across the distribution range of *Calceolaria polyrhiza*. (A) Components of variance (CV) expressed as percentages of total variance among populations, among individuals within populations, and within individuals (error term). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. (B) Among-population CV/within-population CV ratio, showing main source of variation (local versus geographical) for each trait measured. Traits related to floral fit are indicated. Codes for the different morphological variables are provided in Materials and methods and in Fig. 2.

Climatic and edaphic factors

Environmental conditions were highly variable across the distribution range of *C. polyrhiza*. For example, annual temperature and rainfall ranged from 4·6 to 13·7 °C and from 141 to 1033 mm, respectively, whereas soil N and C content ranged from 0·03 to 0·79 % and from 0·27 to 11·47 %, respectively (Supplementary Data Table S1). The climatic variables obtained were summarized into two climatic factors, the first axis of two independent principal component analyses performed with temperatures and precipitation variables, respectively. The temperature factor accounted for 70 % of the total temperature variation and was mainly explained by mean annual temperature and mean temperature of the warmest quarter; the precipitation factor accounted for 86 % of the total variation in precipitation and was mainly explained by mean annual precipitation. Edaphic variables were summarized into two edaphic factors, the first and second axes of the principal component analysis, which accounted for 63 % of the total edaphic variation and were mainly explained by N and C concentration ('soil nutrient', axis 1) and percentage of sand/silt and potassium ('sand/silt', axis 2).

Pollinators

The pollinator functional trait HML was significantly associated with latitude, with HML increasing to the south ($R^2 = 0.48$, $P < 0.0001$). Nested ANOVA indicated that the greatest proportion of variance (CV = 90 %) of bee size was explained by differences between pollinator species ($F = 464.32$; $P < 0.0001$), but variation among populations within bee species

(CV = 2 %) was also significant ($F = 1.97$, $P = 0.01$; $P = 0.02$).

Multivariate patterns and possible factors of floral variation

The RDA that analysed the association between floral traits and environmental variables showed that the pollinator functional trait ($F = 7.42$, $P = 0.001$), precipitation axis ($F = 4.49$, $P = 0.005$) and soil nutrients ($F = 3.25$, $P = 0.03$) were the biotic and abiotic factors contributing significantly to the model (Fig. 7A). The vector representing the direction of maximum change for each explanatory variable showed that the pollinator functional trait (HML) was strongly correlated to the first axis, with populations characterized by flowers with longer filaments and styles and operative distance (ThroL, Fig. 7A) being associated with longer bees. On the other hand, precipitation and soil nutrients were correlated with the second axis, with populations with flowers characterized by larger corollas but smaller elaiophores (Fig. 7A) inhabiting humid and rich-soil sites (mainly located in the north-western area of the distribution range). The RDA that analysed the association between vegetative traits and environmental variables showed that soil nutrients ($F = 9.27$, $P = 0.001$), temperature ($F = 5.00$, $P = 0.003$) and precipitation ($F = 2.83$, $P = 0.035$) were the factors that contributed significantly to the model (Fig. 7B). The vectors representing the direction of maximum change for each explanatory variable showed that mainly soil nutrients and temperature were correlated with the first axis, with populations characterized by higher and larger plants, higher inflorescences, larger leaves and higher SLA values, being associated mainly with rich soils and warm sites (both conditions mainly occurring in the
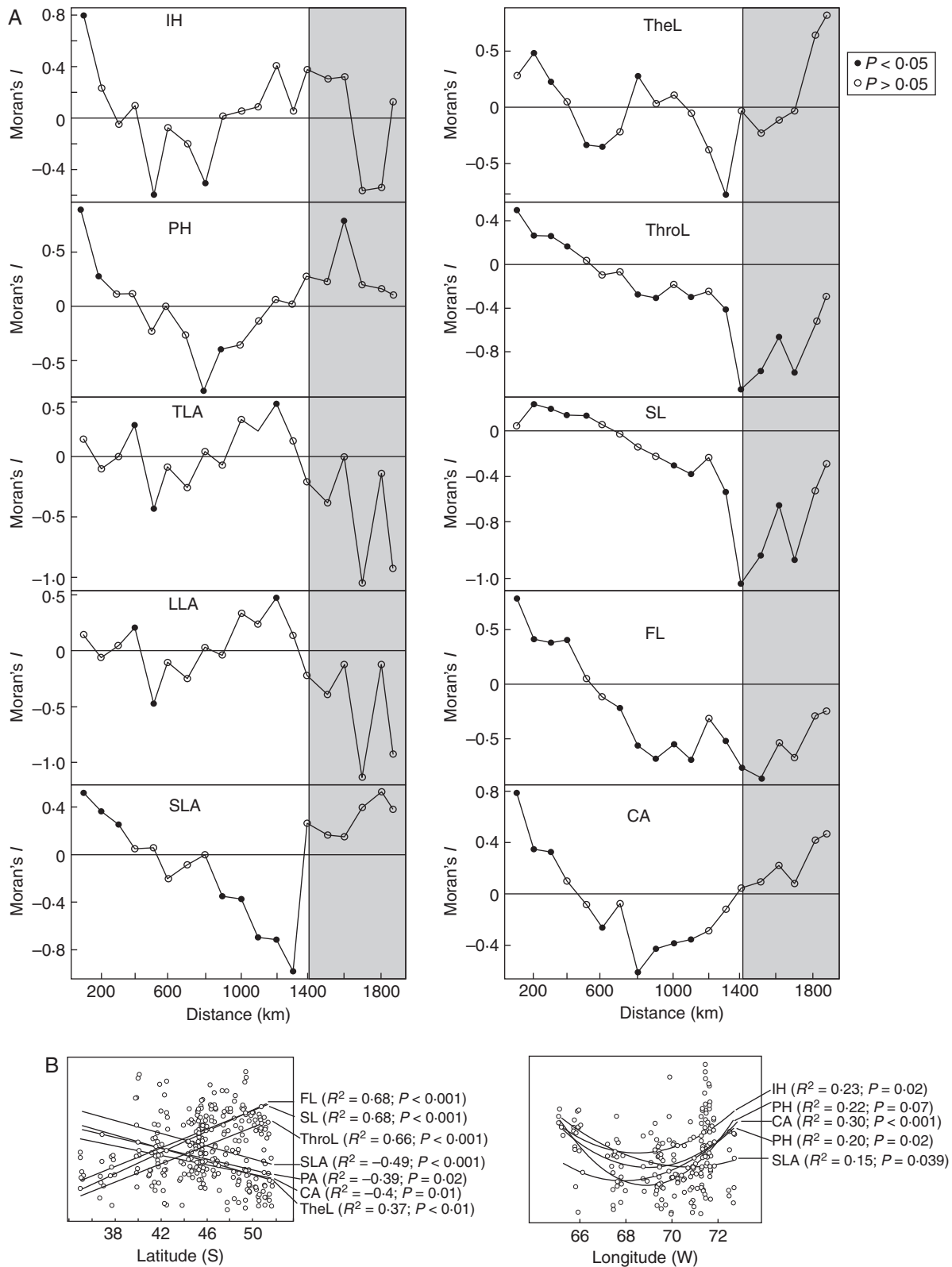
FIG. 5. (A) Spatial autocorrelograms of Moran's *I* coefficient for ten floral and vegetative traits as a function of geographical distance among *Calceolaria polyrhiza* populations in Patagonia. Solid symbols are coefficients that differ significantly from zero; open symbols are non-significant coefficients. The shaded area indicates distance classes that should not be considered because they included fewer than ten pairs of points. (B) Geographical patterns of variation in floral and vegetative traits in 45 populations of *C. polyrhiza*. Only significant relationships are shown. Codes for the different morphological variables are provided in Materials and methods and Fig. 2.
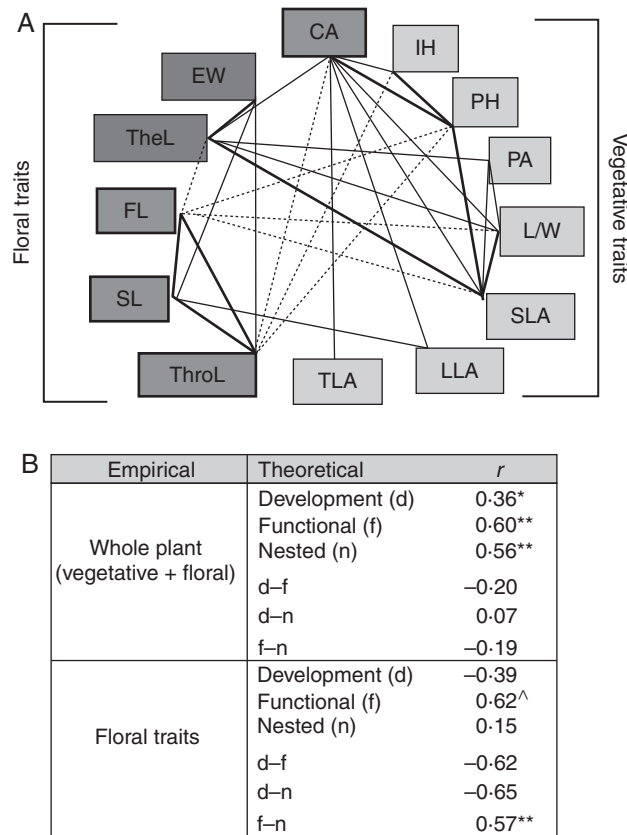
Fig. 6. Phenotypic covariation analysis. (A) Phenotypic correlations among traits measured. Positive correlations among traits are represented by solid lines connecting the traits; line thickness indicates the overall magnitude of each correlation. Dashed lines indicate negative correlations between traits. Floral mechanical-fit-related traits and corolla area are indicated. Codes for the different morphological variables are provided in Materials and methods and Fig. 2. (B) Results of Mantel test (r) indicating similarity between the empirical matrices (vegetative + floral traits or floral traits alone) and the respective theoretical matrices (development, functional and nested). Differences between theoretical matrices (d−f, d−n and f−n) in the power to explain the empirical matrix are also shown. ** $P < 0.01$, * $P < 0.05$, ^ $P = 0.05$.

north-western area of the distribution range). On the other hand, the second axis shows populations with higher SLA values associated with warmer sites (Fig. 7B).

Plant–pollinator matching

Mean values of ThroL for the *C. polyrhiza* populations and the pollinator functional trait H-ML increased to the south (Fig. 8A) and were significantly correlated with each other ($R = 0.90$, $P < 0.0001$; Fig. 8B). The multiple regression analysis that included pollinator length, climatic and edaphic parameters and CA as predictor variables showed that pollinator length remained the main predictor of ThroL ($R^2 = 0.85$, $P < 0.0001$; Table 1). Interestingly, in the multiple regression analysis of CA, which included pollinator length, climatic and edaphic parameters and ThroL as predictor variables, precipitation was the only variable included in the model ($R^2 = 0.43$, $P < 0.05$; Table 1).
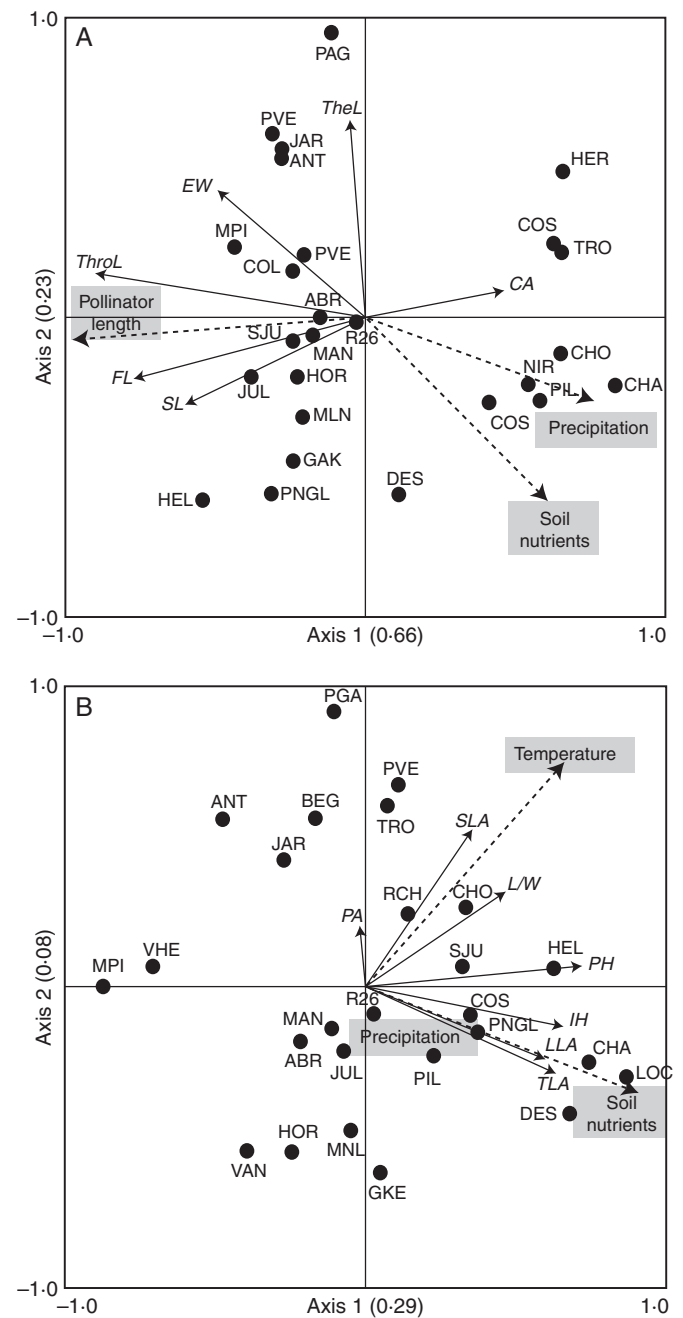
Fig. 7. Biplot of the first two axes of the RDA ordination for 25 *Calceolaria polyrhiza* populations (black circles). The explanatory environmental factors (dashed arrows) that were significant ($P < 0.05$) determinants of floral (A) and vegetative (B) morphological variation (filled arrows) are shown. The eigenvalue associated with each axis is provided in parentheses. The explanatory variables are described in Materials and methods and their values are reported in Table S2. Population acronyms are given in Appendix 1.

DISCUSSION

Our results show that climatic and edaphic conditions are highly variable across the distribution range of *C. polyrhiza*. Additionally, pollinators varied geographically and differed significantly in traits related to the pollination mechanism.

Therefore, these characteristics make this plant–pollinator system appropriate for the exploration of the relative influences of biotic and abiotic factors on floral trait variation at a broad geographical scale.

In correlation with this heterogeneous environmental context, *C. polyrhiza* showed significant intraspecific variation in floral and vegetative traits. In all cases, this variation was significant at the population level, and for most of the traits measured the relationships between the intra- and interpopulation variance

Fig. 8. (A) Geographical variation in the functional trait throat length of *Calceolaria polyrhiza* and in bee length of its pollinators, *Centris cineraria* and *Chalepogenus caeruleus*, in 25 populations. Values of these traits are proportional to the size of the symbols. (B) Relationship between throat length of *C. polyrhiza* and bee length of its pollinators.

component suggests a predominance of geographical factors in shaping spatial patterns of floral and vegetative variation. Interestingly, the floral trait ThroL (operative distance), which plays a critical role in the pollination mechanism, was the variable with the highest amount of interpopulation variation relative to the local variation (i.e. intrapopulation level), which coincides with the spatial scale at which the pollinator shift occurs.

Several evolutionary processes, operating together, may be involved in the phenotypic variation across the distribution range of a species; a random process such as genetic drift being associated with a stochastic interpopulation variation pattern and a non-random process such as natural selection, which generates clinal or mosaic-like geographically structured phenotypic patterns, being associated with spatial changes in biotic or abiotic factors (Grant, 1991). On the contrary, gene flow prevents phenotypic differentiation among populations (Grant, 1991). When the relative effect of gene flow decreases with increasing geographical distance, a distance-based pattern could be the result of isolation by distance. According to the autocorrelation analyses, floral and vegetative characters (except EW, PA and L/W) showed distance-based patterns of significant positive autocorrelation among populations located at distances shorter than 500 km and of negative spatial autocorrelation at distances longer than 500 km. Under a scenario of isolation by distance and under a hypothesis of interpopulation differentiation because of neutral processes (i.e. gene flow and genetic drift), we should observe only a positive autocorrelation at short distances (because of the predominance of gene flow over genetic drift) and non-significant autocorrelation at longer distances (because of the predominance of genetic drift over gene flow; Legendre and Legendre, 1998). By contrast, our results show that populations located more than 500 km from each other are significantly dissimilar in phenotype, suggesting the effect of opposite selective processes. Interestingly, the 'area of influence' of each ecoregion and the distribution range of each pollinator species is about 500 km, so at about that distance the most pronounced environmental (biotic and abiotic) shift occurs. Moreover, the pattern found in the vegetative trait SLA and the floral traits CA, SL, FL and ThroL clearly responds to an environmental gradient, because the correlogram is monotonically decreasing and nearly all autocorrelation values are significant (Legendre and Legendre, 1998). In agreement with these results, several floral and vegetative traits exhibited clinal patterns of geographical variation, being associated with pollinator functional traits, climatic and/or edaphic factors (see below).

We hypothesized that, across the geographical range, floral traits would be decoupled from each other, and that some floral
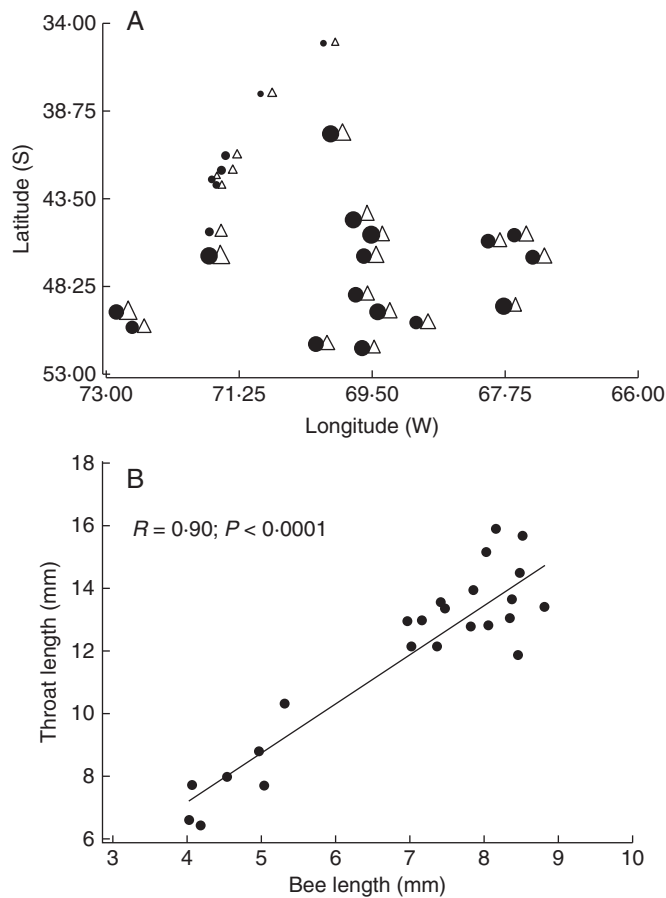
TABLE 1. *Multiple regression models that test the effect of functional bee length (HML), together with allometric and environmental predictor variables (T, temperature; PRE, precipitation; soil nutrients and sand/silt percentage) on throat length and corolla area of* Calceolaria polyrhiza *flowers*

| Response variable | Standardized partial regression coefficient | | | | | | | Model | | |
	ThroL	CA	HML	T	PRE	Soil nutrients	Sand/silt	R^2	F	P
ThroL	–	−0·01	1·58***	−0·36*	−0·38	−0·23	0·001	0·85	18	<0·0001
CA	−0·01	–	0·27	−0·18	−1·27*	−0·54	−0·01	0·43	2·91	0·04

*P < 0·05, ***P < 0·0001.

traits would even be coupled to vegetative geographical variation. Previous studies have explored coupling/decoupling between vegetative and floral characters at a geographical scale (e.g. Herrera *et al.*, 2002, Lambrecht and Dawson, 2007; Perez-Barrales *et al.*, 2007; Chalcoff *et al.*, 2008), considering decoupling as evidence of different selection regimes influencing floral and vegetative traits. Probably because of the traditional idea that pollinator-mediated selection favours covariation of the whole floral phenotype (Berg, 1960; Armbruster *et al.*, 1999), the degree of within-flower coupling/decoupling at a geographical scale has been less explored (but see Rosas-Guerrero *et al.*, 2011). Our results show that throat, filament and style length, which are the critical flower–bee fitting traits involved in the pollination mechanism, were significantly more correlated among each other than with the other floral traits, suggesting the existence of one such functional intrafloral module. These traits increased southwards, where populations pollinated by the larger bee (*C. cineraria*) are located. Interestingly, other floral traits, such as theca length and corolla area, and the vegetative traits plant area and specific leaf area showed the opposite patterns, decreasing southwards, where *C. polyrhiza* inhabits sites under stressful moisture and soil nutrient conditions.

The geographical patterns detected for plant height and area, leaf area, specific leaf area and corolla area were consistent with previous studies showing a reduction in flower size, leaf area and specific leaf area with increasing aridity (e.g. Jonas and Geber, 1999; Sapir *et al.*, 2002; Herrera, 2005; Lambrecht and Dawson, 2007; Paiaro *et al.*, 2012) and soil nutrient availability (e.g. Frazee and Marquis, 1994; Herrera, 2005; Paiaro *et al.*, 2012). Therefore, the reduction in leaf and flower size could be related to strategies for reducing evapotranspiration and reproductive costs in stressful areas, as suggested (e.g. Carroll *et al.*, 2001; Herrera, 2005, 2009). Under certain conditions, this transition to smaller flowers could also be associated with increasing levels of autogamy, as reported for other species (e.g. Pauw, 2005). We suggest that decoupling among floral traits in *C. polyrhiza* may resolve the conflict between limitations in resource allocation to reproductive and vegetative costs and the demands of fitting to a large pollinator inhabiting stressful localities. Thus, in populations located in stressful areas (which are pollinated by the larger bee), *C. polyrhiza* would minimize reproductive costs by reducing floral size (i.e. corolla area) while maintaining covariation patterns among fit-related floral traits for effective pollination. This suggested mechanism probably explains why this highly pollinator-specialized species has a wide geographical distribution (Pélabon *et al.*, 2013; Hermant *et al.*, 2013).

Based on RDA results, and in agreement with the geographical variation pattern of each trait, both biotic and abiotic factors shape floral variation at a spatial scale. However, in the multiple regression analysis of ThroL (operative distance), which included all biotic and abiotic factors as predictor variables, the pollinator functional trait remained the main predictor variable. Thus, ThroL, which has a critical role for an effective pollination mechanism, was strongly associated with bee length, even when other environmental variables were considered simultaneously in the multiple regression models. Interestingly, when this analysis was performed on corolla area, precipitation axis was the only variable retaining explanatory power in the model. In systems where the variation induced by pollinators and abiotic factors have the same direction, it is difficult to determine the contribution of each factor to the phenotypic variation at a geographical scale, because a better fit to the morphology of pollinators may just be the result of an increase in flower size (i.e. isometric change) as a consequence of favourable climatic or edaphic conditions (e.g. Anderson and Johnson, 2008; Hodgins and Barrett, 2008). The geographical structure of biotic and abiotic variation across the distribution range of *C. polyrhiza* provides a scenario in which the expected patterns of either pollinator-driven variation and variation driven by cost–resources trade-offs (Galen, 1999; Herrera, 2005) have opposite directions. Indeed, our results show the simultaneous influences and relative importances of biotic and abiotic factors in the configuration of floral variation at a geographical scale. While pollinators appear to be the main agents of among-population floral variation influencing mechanical-fit-related traits, precipitation seems to be the most important factor influencing corolla area. We consider that geographical studies exploring floral variation related to pollinators should include not only pollination-related traits but also other floral and vegetative variables to thoroughly explore the relative importance of pollinators and abiotic factors as drivers of floral diversification.

Our study, which is the first report on an oil–flower/oil–bee interaction at far southern latitudes in South America, supports the Grant–Stebbins model of pollinator-driven divergence (Johnson, 2010; Peter and Johnson, 2014; Sun *et al.*, 2014; Van der Niet *et al.*, 2014). Overall, by covering the entire distribution range of *C. polyrhiza*, we could detect two floral ecotypes mainly differentiated by floral traits related to pollinator fit, with the pollinator functional trait being the main factor influencing floral divergence. In this system, local adaptation would result from a variable adaptive landscape determined by geographically differentiated ranges of pollinators. The next step in this study system is to analyse the reported floral divergence considering phylogeographical patterns of *C. polyrhiza* (Cosacov *et al.*, 2010) to disentangle historical and ecological processes influencing floral evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Table S1: number, code, name and geographical co-ordinates of *Calceolaria polyrhiza* sampled populations for interpopulation phenotypic variation. Table S2: climatic and edaphic conditions of the 25 *Calceolaria polyrhiza* populations selected in Patagonia. Fig. S1: theoretical development, functional and nested matrices used to analyse coupled/decoupled geographic morphological variation. Fig. S2: geographic patterns of variation of floral and vegetative traits in 45 populations of *Calceolaria polyrhiza*.

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APPENDIX 1. *Population mean (± s.d) values of floral traits measured for 45 populations of* Calceolaria polyrhiza *in Patagonia, southern South America. Locality number, code and number of individuals sampled (*n*) are given. Localities (*N_{loc}*) are numbered consecutively, as shown on the map in Fig. 1. Codes for the different morphological variables are provided in Materials and methods and Fig. 2.*

N_{loc}	Code	Population	n	EW			TheL			FL			SL			ThroL			CA		
1	VHE	Valle Hermoso	30	5·18	±	0·55	3·25	±	0·37	1·86	±	0·22	1·80	±	0·13	6·71	±	0·78	324·67	±	49·00
2	EPU	Epulaufquen	10	6·17	±	0·79	3·73	±	0·30	1·60	±	0·38	1·75	±	0·13	5·90	±	0·64	307·10	±	63·61
3	TRO	Trolopé	30	4·40	±	0·58	3·46	±	0·35	1·73	±	0·19	1·53	±	0·18	7·81	±	1·04	368·67	±	80·47
4	LOL	Lolog	20	4·73	±	0·57	3·56	±	0·42	1·46	±	0·23	1·89	±	0·33	8·08	±	0·82	350·95	±	93·89
5	PAG	Piedra del Àguila	30	7·08	±	0·63	4·35	±	0·36	1·87	±	0·34	2·32	±	0·19	14·47	±	1·44	492·19	±	80·92
6	COS	Colonia Suiza	28	4·34	±	0·56	3·17	±	0·32	1·63	±	0·20	1·88	±	0·20	7·79	±	0·88	364·90	±	100·30
7	MGN	Mogote Nevado	10	4·60	±	0·58	3·45	±	0·36	1·41	±	0·19	2·01	±	0·20	7·25	±	0·81	234·23	±	170·23
8	PIL	Piltriquitrón	42	4·75	±	0·76	3·51	±	0·55	1·73	±	0·21	2·03	±	0·47	8·87	±	1·03	480·71	±	175·69
9	CHO	Cholila	51	3·77	±	0·56	2·97	±	0·39	1·69	±	0·24	1·82	±	0·23	8·06	±	1·04	357·30	±	85·03
10	CHA	Pricho	11	5·21	±	0·66	3·97	±	0·39	1·24	±	0·17	2·62	±	0·44	6·54	±	0·48	558·48	±	119·60
11	VIN	Lago Vintter	31	4·26	±	0·54	3·41	±	0·25	1·80	±	0·19	1·80	±	0·16	7·43	±	0·82	460·62	±	94·95
12	LCA	Lago Carlota	10	5·15	±	0·96	3·48	±	0·27	1·83	±	0·20	2·35	±	0·23	10·76	±	0·96	474·59	±	94·11
13	LTA	La Tapera	10	4·81	±	0·47	3·34	±	0·22	1·62	±	0·23	2·48	±	0·23	9·69	±	0·68	461·74	±	120·91
14	NIR	Ñirehuao	10	4·89	±	0·42	3·61	±	0·72	1·72	±	0·37	2·26	±	0·32	10·37	±	1·50	435·73	±	123·32
15	PRI	Pico Richards	10	5·33	±	0·56	3·43	±	0·32	1·66	±	0·15	1·96	±	0·26	10·10	±	0·85	487·73	±	99·75
16	LES	Laguna Escondida	10	5·04	±	0·37	3·81	±	0·24	1·56	±	0·10	2·24	±	0·17	8·30	±	1·03	446·55	±	133·83
17	BAL	Balmaceda	10	5·91	±	0·87	3·89	±	0·21	1·72	±	0·19	2·32	±	0·19	9·01	±	1·03	403·02	±	162·20
18	VIH	Vista Hermosa	10	5·42	±	1·01	3·78	±	0·32	1·63	±	0·21	2·31	±	0·19	8·21	±	1·10	503·43	±	176·09
19	ANT	Los Antiguos	31	5·63	±	0·72	4·06	±	0·41	2·40	±	0·22	2·36	±	0·19	15·64	±	1·14	435·24	±	79·80
20	SGE	Sierra Grande	30	5·24	±	0·69	3·66	±	0·24	1·94	±	0·25	2·20	±	0·22	12·26	±	1·34	506·16	±	78·35
22	LOC	Lochiel	26	4·41	±	0·64	3·27	±	0·28	1·74	±	0·26	2·12	±	0·22	10·73	±	1·19	391·38	±	91·40
23	RCH	Río Chico	30	5·75	±	0·71	4·02	±	0·23	1·70	±	0·23	2·20	±	0·19	13·36	±	1·38	354·27	±	62·46
24	BEG	La Begonia	27	5·69	±	1·00	3·84	±	0·46	1·91	±	0·32	2·40	±	0·36	12·82	±	1·70	381·82	±	81·95
25	R26	Ruta 26	30	4·84	±	0·85	3·44	±	0·50	2·08	±	0·29	2·29	±	0·25	12·16	±	1·79	382·91	±	75·88
26	MPI	Manos Pintadas	50	6·93	±	0·86	4·11	±	0·40	2·07	±	0·41	2·48	±	0·30	13·05	±	2·04	467·59	±	85·90
27	MAN	Los Manantiales	16	6·45	±	1·06	4·04	±	0·46	2·18	±	0·45	2·36	±	0·20	13·41	±	1·75	504·72	±	88·35
28	PVE	Pampa Verdun	30	6·08	±	1·05	3·87	±	0·59	2·23	±	0·33	2·51	±	0·32	13·94	±	1·50	473·93	±	153·01
29	SUM	Sumich	30	4·80	±	0·56	3·31	±	0·30	2·50	±	0·34	2·49	±	0·23	14·98	±	1·09	478·64	±	80·30
30	COL	Caleta Olivia	29	4·52	±	0·53	3·61	±	0·29	1·83	±	0·24	2·05	±	0·18	13·55	±	1·83	412·56	±	74·70
31	JAR	Jaramillo	30	6·66	±	1·00	4·21	±	0·44	2·16	±	0·24	2·32	±	0·17	15·13	±	1·40	594·17	±	271·14
32	DES	Puerto Deseado	18	4·03	±	0·60	3·00	±	0·27	2·19	±	0·26	2·45	±	0·39	12·96	±	1·35	305·99	±	58·05
33	TMK	Tamel Aike	30	5·15	±	0·57	3·27	±	0·25	2·47	±	0·25	2·48	±	0·23	13·33	±	1·31	492·35	±	85·19
34	GGR	Gob. Gregores	30	5·61	±	0·56	3·23	±	0·24	2·28	±	0·16	2·61	±	0·33	14·24	±	1·02	464·65	±	60·06
35	ABR	Ea. 1 ° Abril	42	4·45	±	1·03	3·57	±	0·52	1·92	±	0·47	2·17	±	0·23	12·79	±	2·17	319·29	±	106·73
36	JUL	La Julia	30	4·88	±	0·61	3·19	±	0·21	2·43	±	0·18	2·44	±	0·19	13·64	±	1·63	431·06	±	63·08
37	SJU	San Julián	30	4·67	±	0·75	2·94	±	0·36	2·16	±	0·29	2·36	±	0·24	11·90	±	1·43	408·52	±	39·87
38	MLN	Monte León	30	5·10	±	0·69	3·16	±	0·24	2·46	±	0·28	2·45	±	0·20	13·00	±	1·30	425·74	±	84·76
39	TRL	Tres Lagos	27	5·30	±	0·71	3·29	±	0·31	2·50	±	0·33	2·52	±	0·26	13·60	±	1·13	520·93	±	99·89
40	HEL	Helsinfors	34	6·45	±	0·73	3·11	±	0·30	2·93	±	0·25	2·85	±	0·33	15·86	±	1·78	641·47	±	141·87
41	PNGL	PN Los Glaciares	49	4·62	±	0·61	2·96	±	0·25	2·22	±	0·33	2·37	±	0·22	12·17	±	1·59	330·64	±	65·72
42	TPK	Tapi Aike	30	5·70	±	0·79	3·18	±	0·29	2·32	±	0·24	2·50	±	0·23	12·79	±	1·61	458·67	±	91·83
43	VAN	La Vanguardia	30	4·99	±	0·70	3·05	±	0·28	2·17	±	0·16	2·29	±	0·23	11·82	±	1·45	378·21	±	75·84
44	HOR	Las Horquetas	50	5·40	±	0·54	3·02	±	0·22	2·26	±	0·25	2·28	±	0·26	12·83	±	1·45	440·68	±	81·31
45	COI	Río Coig	31	5·84	±	0·48	3·19	±	0·21	2·36	±	0·26	2·27	±	0·22	13·05	±	1·03	433·20	±	71·41
46	GAK	Güer-Aike	48	5·48	±	0·98	2·96	±	0·25	2·17	±	0·23	2·35	±	0·27	11·05	±	1·54	359·56	±	79·80

APPENDIX 2. *Population mean (± s.d) trait values of vegetative traits measured for 42 populations of* Calceolaria polyrhiza *in Patagonia, southern South America. Locality numbers and codes are given. Localities (N_{loc}) are numbered consecutively, as shown on the map in Fig. 1. Codes for the different morphological variables are provided in Materials and methods and Fig. 2.*

N_{loc}	Code	TLA	LLA	SLA	L/W	PA	PH	IH
1	VHE	78·5 ± 22·2	67·8 ± 20·7	9473·4 ± 6269·9	2·1 ± 0·4	541·0 ± 576·1	7·1 ± 1·7	5·4 ± 0·8
2	EPU	104·0 ± 20·4	88·5 ± 18·1	8203·4 ± 1340·8	2·3 ± 0·4	NA	NA	NA
3	TRO	129·9 ± 34·2	112·7 ± 30·7	9553·9 ± 2255·7	2·4 ± 0·5	445·6 ± 331·9	15·7 ± 3·4	13·5 ± 3·0
5	PAG	155·4 ± 77·1	146·4 ± 72·3	21745·0 ± 11962·2	3·1 ± 0·7	5095·6 ± 4609·9	20·3 ± 4·2	9·9 ± 2·8
6	COS	154·0 ± 63·7	142·3 ± 57·8	13935·1 ± 4910·8	2·3 ± 0·4	251·5 ± 218·2	20·4 ± 6·6	13·3 ± 4·3
7	MGN	127·5 ± 36·6	116·7 ± 32·3	8520·2 ± 1825·3	2·7 ± 0·5	NA	NA	NA
8	PIL	161·8 ± 88·4	154·3 ± 106·2	13240·3 ± 8864·7	2·7 ± 0·7	8894·0 ± 1924·9	22·2 ± 7·8	16·4 ± 3·8
9	CHO	116·1 ± 45·9	103·8 ± 42·5	16526·7 ± 6418·2	2·5 ± 0·6	358·0 ± 335·3	23·2 ± 6·3	16·3 ± 3·7
10	CHA	267·5 ± 237·7	288·6 ± 274·5	15083·5 ± 4513·3	3·2 ± 0·8	258·1 ± 185·7	36·3 ± 11·8	31·2 ± 10·5
11	VIN	75·9 ± 20·2	68·9 ± 17·4	9648·6 ± 1628·1	2·2 ± 0·3	59·0 ± 64·3	11·4 ± 2·4	10·6 ± 2·3
12	LCA	229·2 ± 106·7	216·7 ± 107·2	13359·9 ± 4053·0	2·8 ± 0·7	NA	NA	NA
13	LTA	187·3 ± 62·9	171·5 ± 59·6	13387·9 ± 3785·5	3·3 ± 0·5	NA	NA	NA
14	NIR	344·0 ± 70·0	314·2 ± 61·7	10893·0 ± 3775·2	3·1 ± 0·7	NA	NA	NA
15	PRI	274·3 ± 127·4	260·0 ± 119·5	10419·1 ± 3530·5	2·8 ± 0·4	NA	NA	NA
16	LES	325·2 ± 119·9	301·4 ± 117·3	18690·7 ± 18344·2	2·5 ± 0·3	NA	NA	NA
17	BAL	217·4 ± 102·5	197·3 ± 97·0	10211·0 ± 1346·9	2·7 ± 0·6	NA	NA	NA
18	VIH	286·0 ± 182·0	266·4 ± 177·4	15440·8 ± 7465·7	3·4 ± 0·6	NA	NA	NA
19	ANT	109·2 ± 37·4	97·2 ± 33·7	13094·8 ± 3719·3	3·0 ± 0·6	570·1 ± 999·6	13·3 ± 2·5	8·6 ± 2·8
20	SGE	267·0 ± 87·8	259·2 ± 84·8	15592·7 ± 12168·6	2·4 ± 0·3	669·8 ± 518·5	19·2 ± 4·8	14·7 ± 3·4
22	LOC	283·2 ± 105·0	257·3 ± 92·7	15922·2 ± 4332·7	3·0 ± 0·6	539·3 ± 499·8	18·3 ± 4·1	12·4 ± 3·4
23	RCH	83·9 ± 29·0	76·6 ± 27·3	12610·3 ± 3025·7	2·9 ± 0·6	122·4 ± 71·4	11·1 ± 2·5	7·7 ± 1·7
24	BEG	81·0 ± 28·0	76·9 ± 28·4	12136·6 ± 2508·1	2·9 ± 0·7	935·1 ± 1273·9	9·2 ± 2·7	3·4 ± 1·1
25	R26	132·2 ± 51·6	122·6 ± 47·6	12123·6 ± 7511·1	2·3 ± 0·5	1668·6 ± 4151·8	10·5 ± 2·8	6·5 ± 2·4
26	MPI	85·5 ± 32·3	83·5 ± 31·6	13226·2 ± 3078·1	2·2 ± 0·4	304·3 ± 282·5	9·4 ± 2·5	4·6 ± 1·4
27	MAN	123·3 ± 42·9	110·8 ± 36·0	11975·5 ± 2686·4	3·2 ± 0·5	1051·8 ± 922·0	15·7 ± 5·2	8·4 ± 1·9
28	PVE	186·4 ± 119·7	173·6 ± 116·9	10860·5 ± 3893·4	2·4 ± 0·6	251·2 ± 254·9	10·6 ± 2·6	8·3 ± 2·3
29	SUM	150·8 ± 49·6	130·5 ± 44·8	7643·4 ± 4112·1	2·3 ± 0·4	371·6 ± 515·2	10·4 ± 1·6	9·2 ± 1·6
31	JAR	132·4 ± 51·9	118·1 ± 46·3	17579·0 ± 7360·1	3·1 ± 0·8	490·2 ± 555·1	14·0 ± 4·0	8·9 ± 2·6
32	DES	256·2 ± 112·0	239·4 ± 93·3	14892·4 ± 3224·9	2·9 ± 0·5	322·7 ± 398·4	17·4 ± 4·2	12·2 ± 2·9
33	TMK	152·2 ± 29·5	143·0 ± 27·1	6634·4 ± 1739·0	1·9 ± 0·2	580·8 ± 492·1	7·2 ± 1·3	6·4 ± 1·1
34	GGR	148·1 ± 40·2	139·8 ± 38·6	8931·1 ± 2128·0	2·0 ± 0·4	138·7 ± 72·1	10·2 ± 2·0	9·1 ± 1·5
35	ABR	81·9 ± 61·0	78·2 ± 59·2	13641·8 ± 4924·7	3·0 ± 0·8	551·4 ± 756·9	8·2 ± 2·7	4·5 ± 1·1
36	JUL	193·3 ± 59·9	185·5 ± 60·0	7420·5 ± 804·2	2·2 ± 0·3	211·2 ± 151·3	13·7 ± 1·9	13·2 ± 1·6
37	SJU	186·9 ± 52·7	172·1 ± 55·0	13708·0 ± 4493·8	2·7 ± 0·5	264·6 ± 209·8	16·0 ± 4·0	14·7 ± 3·7
38	MLN	142·6 ± 46·7	132·2 ± 45·0	7747·0 ± 1325·7	2·2 ± 0·3	126·2 ± 51·7	11·7 ± 2·5	10·6 ± 2·6
39	TRL	189·3 ± 57·2	186·4 ± 51·7	7760·7 ± 1987·0	1·6 ± 0·3	244·1 ± 240·7	9·2 ± 2·2	8·1 ± 2·1
40	HEL	229·8 ± 74·3	214·7 ± 70·4	8202·0 ± 1036·9	2·9 ± 0·5	258·6 ± 275·1	12·9 ± 2·0	11·1 ± 2·4
41	PNGL	124·8 ± 30·7	111·4 ± 28·6	7197·0 ± 844·6	2·3 ± 0·3	85·2 ± 70·5	14·3 ± 3·0	11·4 ± 2·5
42	TPK	165·5 ± 51·5	154·6 ± 47·4	6802·8 ± 744·5	2·1 ± 0·4	585·6 ± 463·3	13·3 ± 2·6	12·5 ± 2·7
43	VAN	166·0 ± 31·9	154·0 ± 29·1	5876·3 ± 663·4	1·9 ± 0·2	374·9 ± 314·6	11·4 ± 1·7	10·4 ± 1·8
44	HOR	192·1 ± 52·2	176·1 ± 49·9	7194·7 ± 859·1	2·1 ± 0·5	223·0 ± 215·7	12·6 ± 2·7	9·5 ± 2·2
46	GAK	168·4 ± 51·1	153·4 ± 48·1	8691·6 ± 1853·6	2·5 ± 0·4	418·2 ± 600·9	13·4 ± 3·1	9·4 ± 2·6

APPENDIX 3. *Population mean (± s.e) values of functional bee length (HML) measured in* Centris cineraria *and* Chalepogenus caeruleus. *Locality code and number of individuals sampled (*N*) are given.*

Code	Bee species	N	HML		s.e.
ABR	*C. cineraria*	1	8·18		–
ANT	*C. cineraria*	2	8·55	±	1·12
COL	*C. cineraria*	6	7·47	±	0·53
CHA	*Ch. caeruleus*	2	4·65	±	0·43
CHO	*Ch. caeruleus*	2	4·65	±	0·43
COS	*Ch. caeruleus*	1	5·14		–
DES	*C. cineraria*	5	7·03	±	0·71
GKE	*C. cineraria*	3	8·26	±	0·68
HEL	*C. cineraria*	2	8·2	±	1·77
HOR	*C. cineraria*	4	8·1	±	0·75
JUL	*C. cineraria*	5	8·41	±	1·32
PNGL	*C. cineraria*	3	7·08	±	0·41
MAN	*C. cineraria*	2	8·84	±	0·21
MLN	*C. cineraria*	4	7·22	±	0·35
MPI	*C. cineraria*	2	8·38	±	0·28
NIR	*Ch. caeruleus*	1	5·41		–
PAG	*C. cineraria*	3	8·51	±	0·98
PIL	*Ch. caeruleus*	3	5·07	±	0·87
JAR	*C. cineraria*	2	8·07	±	0·54
PVE	*C. cineraria*	4	7·9	±	0·53
RCH	*C. cineraria*	1	7·53		–
R26	*C. cineraria*	16	7·42	±	0·66
SJU	*C. cineraria*	2	8·49	±	1·5
TRO	*Ch. caeruleus*	11	4·19	±	0·5
VHE	*Ch. caeruleus*	2	4·15	±	0·64